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Application of solid phase peptide synthesis to engineering PEO–peptide block copolymers for drug delivery

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Abstract

This work describes a simple, versatile solid-phase peptide-synthesis (SPPS) method for preparing micelle-forming poly(ethylene oxide)-*block*-peptide block copolymers for drug delivery. To demonstrate its utility, this SPPS method was used to construct two series of micelle-forming block copolymers (one of constant core-composition and variable length; the other of constant core length and variable composition). The block copolymers were then used to study in detail the effect of size and composition on micellization. The various block copolymers were prepared by a combination of SPPS for the peptide block, followed by solution–phase conjugation of the peptide block with a proprionic acid derivative of poly(ethylene oxide) (PEO) to form the PEO-*b*-peptide block copolymer. The composition of each block component was characterized by mass spectrometry (MALDI and ES-MS). Block copolymer compositions were characterized by ^{1}H NMR. All the block copolymers were found to form micelles as judged by transmission electron microscopy (TEM) and light scattering analysis. To demonstrate their potential as drug delivery systems, micelles prepared from one member of the PEO-*b*-peptide block copolymer series were physically loaded with the anticancer drug doxorubicin (DOX). Micelle static and dynamic stability were found to correlate strongly with micelle core length. In contrast, these same micellization properties appear to be a complex function of core composition, and no clear trends could be identified from among the set of compositionally varying, fixed length block copolymer micelles. We conclude that SPPS can be used to construct biocompatible block copolymers with well-defined core lengths and compositions, which in turn can be used to study and to tailor the behavior of block copolymer micelles.

Keywords: Micelle; Block copolymer; Peptide synthesis; Drug delivery; Nano-engineering

1. Introduction

Amphipathic block copolymers consist of a linear arrangement of alternating hydrophilic and hydrophobic segments. When exposed to polar

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solvents these block copolymers can self-assemble to form extraordinarily stable micellar suspensions consisting of a dense, hydrophobic core and a solvated outer shell [1]. Hydrophobic small molecule drugs can be incorporated into the hydrophobic core during micellization. The amphipathic block copolymer micelle, with its core/shell architecture, and its ability to transport lipophilic substances in aqueous media, is structurally and functionally similar to plasma lipoproteins [2,3]. Thus, researchers are actively investigating the use of biocompatible block copolymers as long-circulating delivery systems for hydrophobic small molecule drugs [2,4,5]. Micellar drug microcontainers can, in principle, impart numerous desirable therapeutic advantages to existing drugs in that they: (a) protect the drug from enzymatic or other degradative mechanisms; (b) increase the solubility of poorly soluble hydrophobic drugs; (c) modulate the drug's pharmacokinetics; and (d) can accumulate at the target site. However, in developing a pharmaceutically useful polymeric micelle for drug delivery, many performance-related issues must be addressed, including drug-loading capacity, release kinetics, circulation time, biodistribution, static and dynamic stability, morphology, size, and size distribution [4]. To a large extent these properties are determined by block copolymer composition. The hydrophilic shell-forming block determines the micelle's pharmacokinetic parameters, biodistribution, and contributes significantly to the physical and biological stability of the micelle. On the other hand, the composition of the core-forming block determines the micelle's drug loading capacity, drug specificity, and contributes largely to the micelle's physico-chemical properties. With a few notable exceptions [6–8], most research on block copolymer micelles for drug delivery has focused on the use of poly(ethylene oxide) (PEO) for the hydrophilic shell. PEO's high degree of hydration and large excluded volume induce repulsive forces that impart steric stability to the micelle, and extend circulation times by preventing opsonization, thereby avoiding clearance by the reticuloendothelial system [9]. In contrast to the near universal application of PEO for the hydrophilic shell-forming block, a somewhat larger variety of hydrophobic core-forming blocks have been investigated. Examples of core-forming block compositions include polycaprolactone [6], poly(D,L-lactide) [5,10], poly(propylene oxide) [11], and poly(L-amino acids) and their derivatives [12–15].

Although biocompatible PEO-*b*-peptide block copolymer micelles show promise as drug carriers, their development and study has been slowed by the difficulty associated with their synthesis. Traditionally, block copolymer synthesis is performed by derivitizing one or both ends of the PEO block with an initiator, and then adding the PEO to a solution containing the monomeric L-amino acid *N*-carboxyanhydride which, upon polymerization, yields the block copolymer [2]. This synthetic route, although reproducible and well established, requires strict control of reagent purity and stoichiometry in order to minimize polydispersity and by-product formation. Moreover, this strategy is restricted to the formation of compositionally homogeneous copolymer hydrophobic blocks. Hence, for a given hydrophobic block composition, micelle nano-engineering is limited to 'approximately' adjusting the relative and absolute lengths of the block copolymer segments.

We have hypothesized that the techniques of solid phase peptide synthesis (SPPS) may be adapted to the construction PEO-*b*-peptide block copolymers with precisely defined core lengths and core compositions, thus allowing the properties of the resulting micelles to be engineered in fine detail. To test this hypothesis, we used SPPS, followed by solution phase condensation (SPC), to prepare two series of block copolymers: one of fixed composition and varying length; the other of fixed length and varying composition. We then performed experiments to systematically study the relationships of core length and core composition to micelle size and thermodynamic stability in terms of free energy of micellization (CMC), micelle-unimer equilibrium, and micelle dissociation rates.

2. Materials and methods

2.1. Materials and chemicals

Five thousand molecular weight α-methyl-ω-proprionic acid–PEO (PEO–O–CH_2–CH_2–COOH) was obtained from Shearwater Polymers Inc. (Huntsville, AL). Rink amide methylbenzhydrylamine resin (Rink Amide MBHA), *O*-benzotriazole-*N*,*N*,*N*′,*N*″-tetramethyluronium hexafluorophosphate (HBTU), piperidine, and Fmoc-amino acids were purchased from Chem Impex International Inc. (Woodale, IL) and used as received. HATU ((7-azabenzotriazol-1yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate) was purchased from PerSeptive Biosystems (Framingham, MA). Sequencing grade dimethylformamide (DMF) and dichloromethane (DCM) were obtained from Fisher Scientific Canada. Trifluoroacetic acid (TFA) was purchased from Halocarbon Products Corporation (River Edge, NJ). *N*-Methyl morpholine (NMM) was purchased from Aldrich Chemical Co. (Milwaukee, IL). Doxorubicin hydrochloride (DOX) was purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. PEO–proprionic acid characterization

PEO–proprionic acid was characterized for weight-average molecular weight (M_w), number average molecular weight (M_n,) and polydispersity index (I) by MALDI-TOF mass spectrometry. A 200 μM sample of PEO–proprionic acid in ddH_2O was prepared and mixed 1:1 with a matrix solution consisting of 0.1 M dihydroxybenzoic acid dissolved in 1:1 MeOH and doped with 2 mM NaCl to promote ionization. Spectra were acquired on a PerSeptive Biosystems Voyager Elite MALDI-TOF mass spectrometer. M_w: 5223, M_n: 5180, *I*: 1.01.

2.3. Solid-phase peptide synthesis, cleavage, and purification

A series of poly L-amino acid (PLAA) core-forming blocks with the composition Gly-Tyr(n) (where $n = 7$, 9, 12, and 15 residues), and a series of constant length blocks with the compositions Gly-Phe_{15} (polyphenylalanine), Gly-Leu_{15} (polyleucine), and Gly-Phe-Leu-Tyr-Trp-Phe-Leu-Tyr-Trp-Phe-Leu-Tyr-Trp-Phe-Leu-Tyr (polyFLYW), were assembled using an ABI 430A automated peptide synthesizer on Rink amide MBHA resin using standard Fmoc/*t*-Butyl protected amino acids and HATU active-ester based coupling [16]. Upon completion of each peptide block synthesis the protected peptide–resin was transferred to a 20 ml disposable polypropylene cartridge fitted with a polyethylene frit. A 10 ml solution of TFA–H_2O (95:5) cleavage cocktail was added and the mixture shaken gently for 2 h. The cleaved and deprotected peptide block was then filtered from the resin and the resin washed with 3×2 ml of TFA. The combined eluate was evaporated in vacuo, precipitated and washed with cold ether (3×20 ml), then dried in vacuo overnight to prepare for purification.

Each crude peptide was purified by RP-HPLC (Waters-System 501) on a reversed-phase C_8 21 × 250 mm Zorbax 300 S.B. column with a binary gradient at a flow rate of 8.0 ml/min using aqueous 0.1% TFA and 0.1% TFA in acetonitrile as the mobile phases. The eluent was monitored at 220 nm and 1-min fractions were collected. UV absorbing fractions were analyzed by mass spectrometry using a Fisons VG Trio 2000 electrospray mass spectrometer (ES-MS). Fractions containing the correct mass and of sufficient purity were combined and lyophilized to yield the desired peptide block in excess of 90% purity, as judged by ES-MS analysis.

2.4. Block copolymer synthesis

PEO-*b*-peptide block copolymers were prepared using solution phase condensation (SPC). PEO–proprionic acid was coupled to each peptide block in solution at a ca. 0.05 mmol scale. The PEO–proprionic acid carboxyl group was activated using a 0.9 molar equivalent of HATU and two molar equivalents of NMM in DMF. The PEO–proprionic acid was allowed to activate for 1 h at room temperature on a shaker. The activated PEO (2 molar equivalents with respect to the hydrophobic block) was then coupled to the glycine N-terminal amine of the peptide block in DMF until

a negative Kaiser test [17] was obtained (overnight at room temperature on a shaker). The crude block copolymer was dialyzed with three changes of DMF (overnight at room temperature with stirring using Spectra/Por 3500 MWCO dialysis membrane) to remove any unreacted peptide and small molecular weight contaminants. DMF was then removed from the polymer by evaporation in vacuo. Unreacted PEO was removed from the block copolymer by preparative HPLC. The UV absorbing fractions were collected, lyophilized, desiccated, and stored at 4 °C. The segment condensation coupling efficiency was determined by ^{1}H NMR analysis (relative integrated peak area) of the block copolymer in DMSO-d_6.

2.5. Determination of CMC

The critical micelle concentration (CMC) was determined by light scattering according to a previously published procedure [18]. Block copolymer micelles of each of the seven different PEO-*b*-peptide block copolymers were prepared by a gradient dialysis approach [19]. The block copolymer (20 ml) was dissolved in DMF (5 ml) and 10 ml of ddH_2O was added dropwise over 2 h. The solution was dialyzed into three changes of 1 l ddH_2O over 24 h. The micelle solution was filtered through a 0.22 μm nylon membrane (Fisher, Pittsburgh, PA) and lyophilized. Lyophilized micelles (12 mg) were accurately weighed and dissolved into 12 ml 0.1 M PO_4^-, pH 7.2. A series of doubling dilutions, from 1 to 1×10^{-4} mg/ml, were prepared in triplicate from this stock solution. Light scattering was measured with a SPEX Fluoromax spectrofluorometer using excitation and emission wavelengths of 600 nm, a bandpass width of 1 nm, a step increment of 0.5 nm, and an integration time of 1.0 s. The intensity of scattered light was plotted against polymer concentration to determine the concentration at which the intensity sharply increases, indicating the formation of micelles.

2.6. Electron microscopy examination

Lyophilized micelles were reconstituted in ddH_2O (ca. 1 mg/ml). Samples of the aqueous micelle suspensions were examined by negative-stain transmission electron microscopy (TEM). One drop of the micelle sample was placed on a 300-mesh copper membrane coated disk, followed by one drop of 1% phosphotungstic acid in water (pH 7.0) (the negative stain). After 30 s excess liquid was blotted from the disk with filter paper and the sample loaded onto a sample holder. The sample was then examined using a Hitachi Transmission Electron Microscope H-7000 at an accelerating voltage of 75 keV. Micelle size and size distribution was determined directly from the TEM images.

2.7. Drug loading of micelles

Doxorubicin hydrochloride was loaded into PEO-*b*-polytyrosine$_7$ micelles according to a previously published procedure [20]. DOX (10 mg) and PEO-*b*-polytyrosine$_7$ block copolymer (20 mg) were added to DMF and mixed for 1 h. The solution was first dialyzed against 1:15 v/v DMF:ddH_2O over 18 h, then dialyzed into three changes of ddH_2O over 24 h. The mixture was microcentrifuged for 10 min at 13,000 rpm to remove precipitated doxorubicin, and the supernatant diluted to 10 ml with ddH_2O. Drug loading was determined by gel permeation chromatographic analysis of an aliquot of the micelle solution and by measuring UV absorbance of the eluent at 485 nm using a Pharmacia Biotech Ultraspec 3000 UV–Vis spectrophotometer.

2.8. Micelle dissociation rates

Accurately weighed samples of lyophilized block copolymer micelles were reconstituted in 0.1 M phosphate buffer (pH 7.2) at concentrations above their critical micelle concentration (ca. 1.0–2.0 mg/ml). An accurately measured aliquot was removed and diluted to a concentration below the CMC (from ca. 0.02 to 0.001 mg/ml) and incubated at 37 °C in a dry bath. One hundred microlitres samples were withdrawn periodically and subjected to chromatographic analysis using a Rainin HPLC system consisting of a Rainin HPXL solvent delivery system, a Rainin Dynamax UV–Visible absorption detector. Samples were applied

to an Ultrahydrogel 2000 column and matching guard column at a flow rate of 0.8 ml min^{-1} and 0.1 M phosphate buffer (pH 7.2) as the mobile phase. The column was calibrated with size exclusion standards (blue dextran, 2,000,000 Da; proteins, 600,000–17,000 Da; and sodium azide, 65 Da). The eluent was monitored at an appropriate wavelength for detection of the hydrophobic block.

3. Results and discussion

Although recent reports have emerged describing the construction of PEO-*b*-peptide block copolymers [21], to our knowledge, this is the first reported application of solid phase peptide synthesis specifically for preparing PEO-*b*-peptide block copolymer micelles. Given the ease with which custom peptides can be prepared or purchased (through commercial and non-commercial peptide synthesis facilities), we believe this hybrid SPPS–SPC approach provides a convenient and cost-effective alternative to the conventional NCA-based ring-opening polymerization strategy. Indeed, a person only needs to order peptides and to have access to an HPLC and a few commercial reagents to prepare these block copolymers—they do not have to be peptide chemists.

The key advantages of SPPS over classical polymerization techniques are the ability to apply combinatorial chemistry to the polymer's construction, and the ability to precisely control core length. Hence, the flexibility of nano-engineering micelle-based drug delivery systems can be extended considerably. In the present study, these properties are exploited to study micellization behavior. Other examples of the possible applications of this technology include: incorporation of crosslinking residues (such as cysteine) into the core-forming block to produce rigid cores; construction of multipartite systems with separate blocks for core formation, drug binding, and shell formation; and incorporation of cleavage substrates into the polymer chain for proteolytically enhanced micelle degradation and drug release.

This SPPS technique was used to construct two sets of PEO-*b*-peptide block copolymers: one with varied compositions but fixed core length, and the other of fixed composition (polytyrosine) but of varying core lengths. The variable composition series includes the following block copolymer constructs: PEO-*b*-polyleucine (hydrophobic, aliphatic), PEO-*b*-polytyrosine (hydrophobic, aromatic), PEO-*b*-polyphenylalanine (hydrophobic, aromatic), and PEO-polyFLYW, which is unique among this series, in that the core-forming block contains a compositionally diverse distribution of aliphatic and aromatic residues. It was designed to study the effect of sequence heterogeneity on block copolymer micellization in analogy with globular proteins, which contain a heterogeneous structure and form core/shell type particles with glassy cores under physiological conditions [22]. The variable length series is composed of polytyrosine peptide blocks of 7, 9, 12, and 15 residues. This series was used to examine the effect of core-block size on micellization.

3.1. Synthesis and characterization

A diagram describing structures of the various block copolymer constructs is provided in Fig. 1. Peptide block compositions and lengths were controlled by altering the number of SPPS cycles and the order of addition of protected L-amino acids. All seven peptide blocks were appended at the N-terminus with a single glycine residue to aid in the conjugation efficiency of PEO to the peptide block. Glycine is a relatively hydrophilic, unhindered, amino acid and is known to disrupt secondary structure formation. These properties combine to help expose the N-terminal amino group and therefore increase coupling efficiency [23].

The crude peptide blocks were purified by conventional preparative RP-HPLC. Purity analysis by analytical RP-HPLC was not possible to obtain purity levels in excess of 90%, as judged by ES-MS analysis, for all the constructs prepared in this study (data not shown). The remaining fractions consisted of (typically 1-residue) deletion products; hence, the core-forming blocks prepared by SPPS can be considered for all intents and purposes to be monodisperse. This ability to precisely control peptide block length can in turn

be exploited to tailor micellization properties such as CMC and dissociation rates, as we demonstrate here.

Preliminary attempts in our lab to synthesize the entire PEO-*b*-peptide block copolymer using SPPS resulted in low coupling efficiencies, due to the inability of PEO to effectively penetrate the peptide–resin (unpublished results). Instead, the conjugation of PEO to the peptide was performed by solution–phase condensation of PEO–proprionic acid with the peptide N-terminal amine. The solvents and reagents used in the conjugation step are identical to those used for amino acid coupling during SPPS and result in the efficient formation of a chemically stable amide bond, making this a convenient and attractive conjugation strategy. Coupling of PEO to peptide was complete after 24 h, as measured by the Kaiser ninhydrin assay for free amines [17]. Purification was achieved by dialysis to remove small molecular weight contaminants and unreacted peptide, followed by RP-HPLC to remove any unreacted PEO. ^{1}H NMR

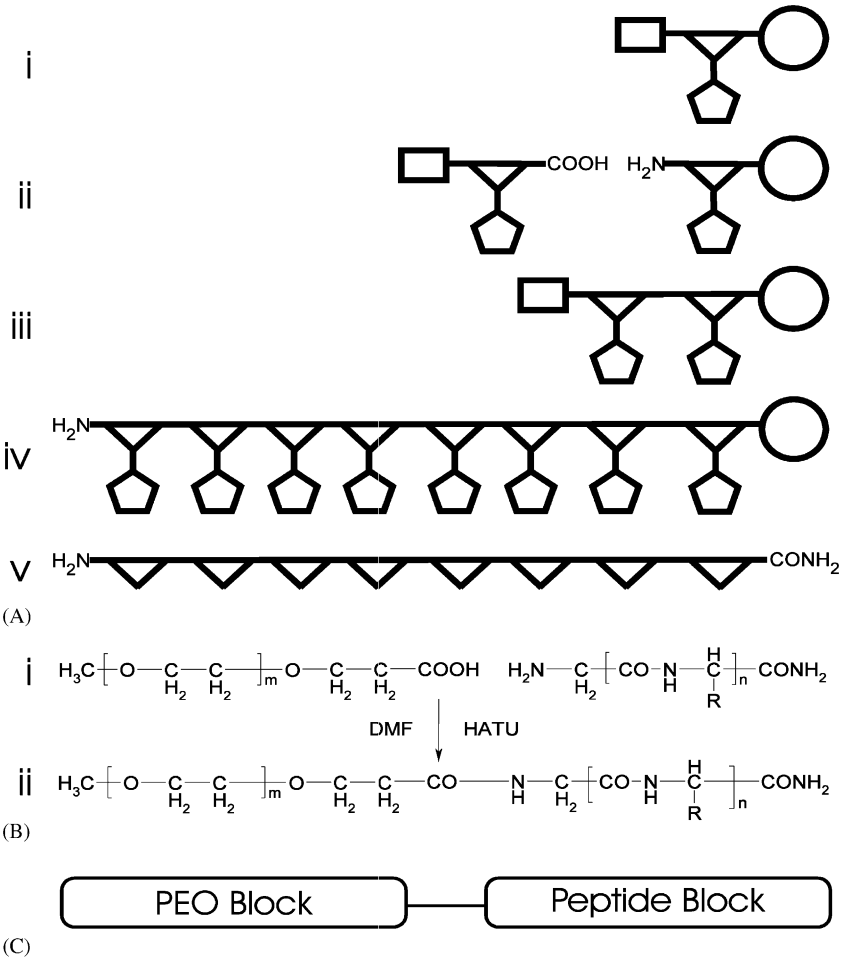

Fig. 1. (A) Schematic diagram depicting the construction of the construction of the peptide block by SPPS: (i) amino acids (triangle), bearing sidechain protection (hexagon) and N-α amino-protection (square) are attached to the insoluble support (circle). (ii) The backbone-protecting group is removed. (iii) The incoming amino acid is activated with HATU and coupled to the growing peptide–resin. (iv) Repeated cycles of deprotection, activation and coupling yield the complete PLAA–resin. (v) The Sidechain protecting groups and the peptide–resin linkage are removed by exposure to TFA. (B) Formation PEO-*b*-peptide: (i) PEO–proprionic acid is condensed to PLAA by amide bond formation (ii). (C) Schematic diagram of the amphipathic block copolymer.

Table 1
PEO-*b*-PLAA block copolymer ^{1}H NMR integration results

Block copolymer	Integration		PEO:PLAA integration ratio		Integration ratio (percent found/expected)
	PEO	PLAA	Expected	Found	
PEO-*b*-polytyrosine$_7$	93.73	6.27	15.53	14.94	96.20
PEO-*b*-polytyrosine$_9$	93.83	6.17	12.88	15.20	118.0
PEO-*b*-polytyrosine$_{12}$	90.77	9.23	9.66	9.83	101.7
PEO-*b*-polytyrosine$_{15}$	89.32	10.68	7.73	8.36	108.2
PEO-*b*-polyphenylalanine$_{15}$	83.62	16.38	6.18	5.11	82.68
PEO-*b*-polyleucine$_{15}$	84.71	15.29	5.15	5.54	107.6
PEO-*b*-G-polyFLYW	96.32	3.68	29.0	26.17	90.24

analysis of the purified PEO-*b*-peptide block copolymer in deuterated DMSO revealed the expected peak intensity ratio of PEO methyl protons at 3.55 ppm to amino acid side-chain protons to within an accuracy of ca. 20% (Table 1).

3.2. Critical micelle concentration

The CMC is the minimum concentration of copolymer that will result in micelle formation. This parameter is an important indicator of the stability of a micelle: the lower the CMC value, the more stable the micelle. Light scattering was used to determine the CMC values of the various PEO-*b*-peptide micelles (Table 2 and Fig. 4). At concentrations below the CMC, the change in light scattering intensity with a corresponding change of concentration is minimal. At and above the CMC, the block copolymer unimers self-assemble into micelles, resulting in a dramatic increase in the sensitivity of light scattering to concentration (Fig. 2). The CMC of the PEO-*b*-peptide constructs is taken as the intersection between the two essentially linear sections of the intensity-concentration curve (Table 2).

The CMC values for the variable length PEO-*b*-polytyrosine block copolymers decreased (indicating greater stability) as the length of the hydrophobic block increased, consistent with theory [24] and observation [25]. The range of values (0.5–0.05 mg/ml) compare favorably with those reported for other PEO-*b*-poly(amino acid) block copolymers under investigation as drug delivery vehicles [26–28].

The relationship between peptide block composition and CMC is not as clear as for peptide block length. The observed CMC values for the fixed length, variable composition series were compared against their predicted hydrophobicity, using the

Table 2
Micelle characteristics

Block copolymer	HPLC elution time (min)		CMC (mg/ml)	Size (nm)	Peptide hydrophobicity[a]
	Unimers	Micelles			
PEO-*b*-polytyrosine$_7$	15.59±0.04	n.d.	0.46	24.5±0.90	0.960
PEO-*b*-polytyrosine$_9$	15.62±0.05	n.d.	0.19	26.3±0.30	0.960
PEO-*b*-polytyrosine$_{12}$	15.60±0.05	12.25±0.02	9.080	26.8±0.40	0.960
PEO-*b*-polytyrosine$_{15}$	15.92±0.09	10.04±0.02	0.050	28.3±0.7	0.960
PEO-*b*-polyphenylalanine$_{15}$	15.74±0.02	n.d.	0.019	38.6±0.3	1.790
PEO-*b*-polyleucine$_{15}$	17.20±0.02	11.501±0.05	0.014	24.0±1.2	1.700
PEO-*b*-polyFLYW	15.61±0.02	n.d.	0.20	63.7±0.5	1.675

[a] Based on Fauchere–Pliska hydrophobicity values.

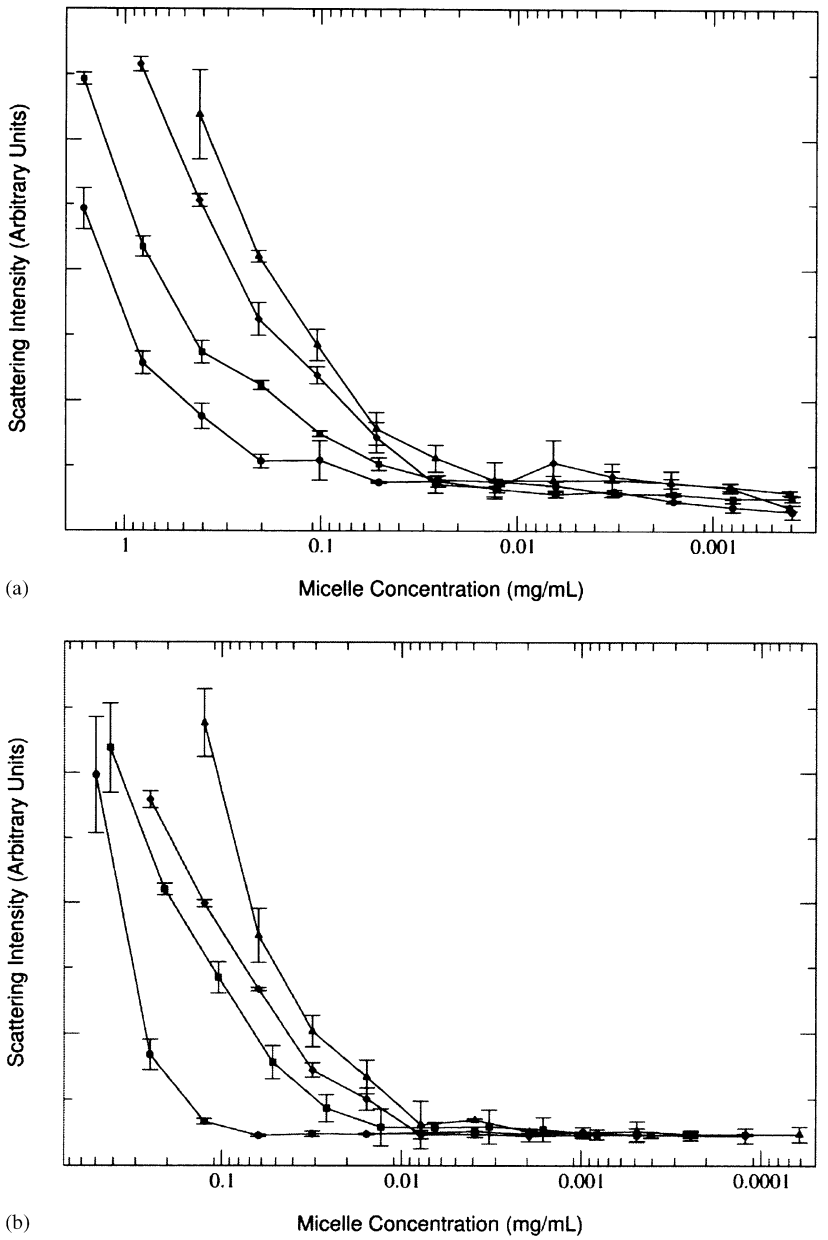

Fig. 2. CMC determination by light scattering: (a) PEO-*b*-polytyrosine with core lengths of 7 (circles), 9 (squares), 12 (diamonds), and 15 (triangles) residues; (b) Fixed core length series (15 residues) with core compositions of polyleucine (triangles), polyphenylalanine (diamonds), polytyrosine (squares), and polyFLYW (circles).

scale of Fauchere and Pliska [29]. The CMC values associated with the homopolymeric PEO-*b*-peptides correlated reasonably with their predicted hydrophobicities; however, the CMC value obtained for the heterogeneous core was strikingly high compared to the value expected from its predicted hydrophobicity. Considering that three of the four amino acids comprising the polyFLYW peptide are represented in the compositions of its homopolymer counterparts, it seems likely that

this discrepancy is due to sequence heterogeneity. Chothia showed that the 'effective' hydrophobicity of peptides and proteins are influenced by their secondary structure, which in turn are influenced by their primary sequence and the polarity of the surrounding medium [30]. Cammas et al. [31,32] studied the conformation of PEO-*b*-poly(β-benzyl L-aspartate) in organic solvents, but did not relate the effect of conformation on micellization thermodynamics. We speculate that the sequence variability within the polyFLYW core translates into a more disordered secondary structure than for the homopolymeric series, and that this disorder results in a greater exposure of the peptide's relatively polar main chain to the surrounding medium, thus decreasing hydrophobicity and increasing the observed CMC.

3.3. Micelle drug encapsulation

The ability of PEO-*b*-PLAA block copolymer micelles to encapsulate model hydrophobic drugs is also an indication of their drug delivery utility. Physical entrapment by dialysis and oil-in-water emulsion methods have both been shown to successfully place hydrophobic drugs, such as the anticancer drug doxorubicin, at high levels within PEO-*b*-PBLA micelle cores without chemical degradation [20] [[33]]. Dialysis was used in a preliminary experiment to encapsulate doxorubicin within PEO-*b*-polytyrosine$_7$ block copolymer micelles. Using UV absorbance and gel permeation chromatography to quantify and determine the proportion of encapsulated versus solvated doxorubicin, a significant drug loading level of 2.8%w/w was found, compared to 14%w/w determined for PEO-*b*-poly(β-benzyl-L-aspartate)$_{12}$ (unpublished results). It is plausible that higher 'solubilities' for this drug could be obtained in the drug loading of PEO-*b*-polyleucine$_{15}$, whose core has a higher predicted peptide hydrophobicity (Table 2). As well, it can be hypothesized that longer core lengths may also aid in increasing drug loading efficiency. The CMC values for the variable length PEO-*b*-polytyrosine block copolymers decreased (indicating experimentally greater stability and hydrophobicity) as core length increased (Table 2), suggesting that PEO-*b*-polytyrosine$_{15}$ would display higher doxorubicin loading than observed for PEO-*b*-polytyrosine$_7$.

3.4. Micelle size distribution

Block copolymer micelle shape, size and size distribution was examined by TEM (Fig. 3). Spherical, uniform micelles, with diameters typical of those reported for other polymeric micelles, were observed for all seven constructs (see Table 2) [7,27,34,35]. Micelle diameters within the size

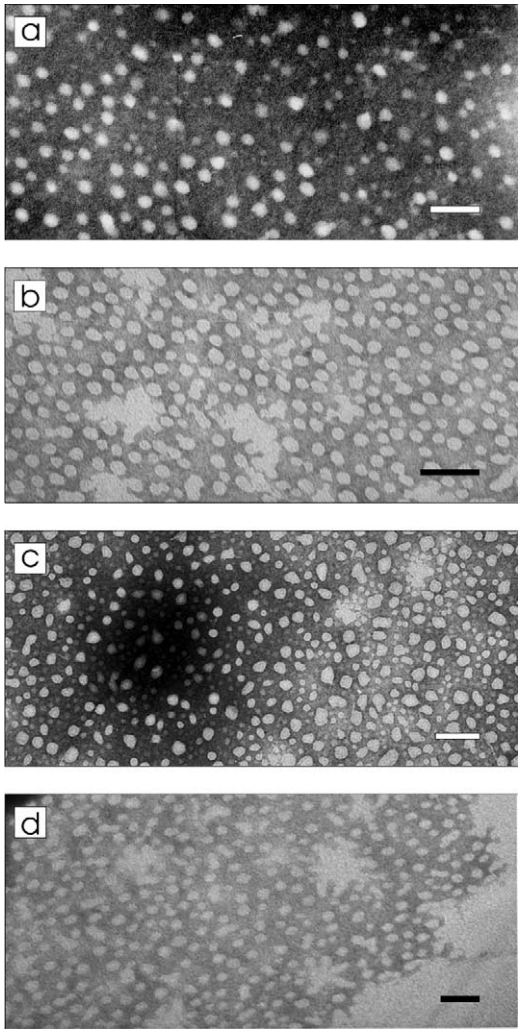

Fig. 3. TEM images for: (a) PEO-*b*-polytyrosine$_7$; (b) PEO-*b*-polytyrosine$_9$; (c) PEO-*b*-polytyrosine$_{12}$; and (d) PEO-*b*-polytyrosine$_{15}$. The scale for all images is 100 nm.

range are considered desirable for drug-delivery; that is, they are large enough to escape renal excretion, yet small enough to avoid hepatic elimination. As seen for other PEO-*b*-peptide block copolymer micelles, this size range, coupled with the non-immunogenic PEO shell, imparts a 'stealthy' quality to the micelle allowing it to circulate in vivo for extended periods of time [13].

Micelle diameters were observed to increase slightly with increasing peptide block length. In contrast, the micelle diameters for the fixed size series were much more varied. The PEO-*b*-poly-FLYW construct showed the greatest deviation, with a diameter over twice the average diameter of the other homopolymeric peptide cores. The reason for this deviation is not clear; however, it seems likely that, as with the CMC, the heterogeneous core composition is a major contributing factor.

3.5. Micelle dissociation rates

A micelle's dynamic stability is an important measure of its utility for drug delivery. Upon introduction to the blood compartment, micelles are diluted to concentrations below the CMC, making them thermodynamically unstable. In contrast to low molecular weight surfactant micelles, which dissociate quickly (on the order of milliseconds), block copolymer micelles have been shown to have much longer dissociation rates at concentrations below their CMC (hours or days) [5,36]. A major factor influencing micelle dissociation rate is the viscosity of the micelle core. ^{1}H NMR studies on bock copolymer micelles with long dissociation rates reveal a solid-like core [37].

Micelle dissociation rates were studied by size exclusion (SEC) HPLC. Using molecular weight markers, the excluded volume was found to occur at 10.02 min (post injection) and the void volume was found to occur at 15.27 min. This information was used to monitor the dissociation of micelles into unimers over time (Table 2 and Fig. 4).

The size varying PEO-*b*-polytyrosine series showed a clear and strong dependence of core length with dynamic stability. Both the seven and nine residue polytyrosine cores dissociated immediately upon dilution below their CMC. At 12 residues, the presence of micelles was observable up to 48 h after dilution. The 15 residue PEO-*b*-polytyrosine demonstrated far greater stability, with less than 30% dissociation after 7 days. These observations are consistent with the idea of a critical molecular weight below which (at a given

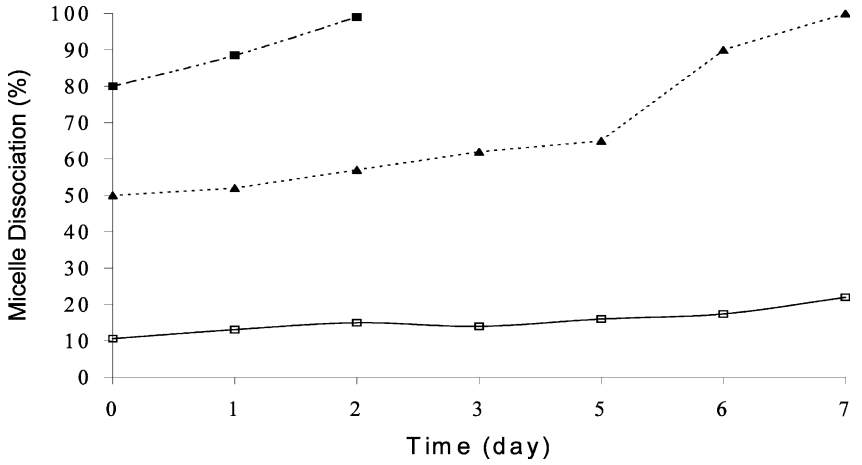

Fig. 4. Dissociation rates for PEO-*b*-peptide micelles PEO-*b*-polytyrosine$_{12}$ (closed squares), PEO-*b*-polytyrosine$_{15}$ (open squares), and PEO-*b*-polyleucine$_{15}$ (closed triangles). Micelle dissociation percent is defined as the ratio of the area under the curve of the block copolymer micelle to that of the free unimer.

temperature) the polymer exists as a melt, and above which the polymer exists in the solid, glassy state. For PEO-*b*-polytyrosine, it appears that a minimum chain-length of 12 residues is necessary to form micelles with a solid-like core.

Of the various fixed-size constructs investigated, only PEO-*b*-polytyrosine and PEO-*b*-polyleucine showed long-term stability below their CMC. Both PEO-*b*-polyFLYW and PEO-*b*-polyphenylalanine were not stable below the CMC. This apparent instability of PEO-*b*-polyphenylalanine is unexpected, considering that the chemical structure of phenylalanine is very similar to that of tyrosine (tyrosine has a *para* hydroxyl group whereas phenylalanine does not), yet the 15-residue PEO-*b*-polytyrosine micelles are the most stable of all the micelles examined in this study. The reason for this discrepancy is not entirely clear, however, it is possible that the increased size of the tyrosine side-chain over phenylalanine coupled with its ability to participate in intermolecular hydrogen bonding may be sufficient to account for the difference in observed dissociation rates. Regardless, these results show that micelle stability is a complex function of core composition, and cannot be predicted on the basis of hydrophobicity alone.

4. Conclusions

Block copolymer micelles hold much potential as non-toxic, non-immunogenic, controlled-release systems for hydrophobic drugs. We have developed a simple and versatile SPPS–SPC method for the construction of PEO-*b*-peptide block copolymers that greatly expands the preparative capabilities of these polymers. To demonstrate its utility and versatility we employed this technique in the construction of a number of PEO-*b*-peptide block copolymers with precisely defined sequence compositions and sizes. This allowed us to investigate the relationship of core size and composition to micelle formation and stability. This work should aid in the nano-engineering and preparation of novel block copolymer constructs for drug delivery.

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